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Thrombomodulin expressing monocytes are associated with low risk features in myelodysplastic syndromes and dampen excessive immune activation

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Abstract

The bone marrow of low risk myelodysplastic syndromes is often associated with an inflammatory environment and active cellular immune response. An active immune response generally contributes to anti-tumor responses and may prevent disease progression. However, chronic immune stimulation can also induce cell stress, DNA damage and contribute in the pathogenesis of myelodysplastic syndromes. Characterizing the protective mechanisms against excessive immune activation is therefore an important aspect of the pathophysiology and may help us to better understand the fine balance between protective and destabilizing inflammation in lower risk disease. In this study we have investigated the role of thrombomodulin (CD141/BDCA-3) expression, a molecule with anti-inflammatory properties, on monocytes in the bone marrow and peripheral blood of myelodysplastic syndrome patients within different risk group. Patient-derived classical monocytes show high expression levels of thrombomodulin, whereas monocytes from healthy donors hardly expressed any thrombomodulin. The presence of thrombomodulin on monocytes from myelodysplastic syndrome patients correlated with lower risk disease groups and a better overall and leukemia-free survival. Using multidimensional mass cytometry (CyTOF), in an *in-vitro* setting, we show that thrombomodulin positive monocytes could polarize naïve T cells toward cell clusters which are closer to Th2 and Treg phenotypes and less likely to contribute in an effective immune-surveillance. In conclusion, the expression of thrombomodulin on classical monocytes is a favorable and early prognostic marker in patients with low risk myelodysplastic syndromes and may represent a new mechanism in the protection against disproportionate immune activation.

Introduction

The immune system plays an important role in the pathogenesis and disease course of myelodysplastic syndromes (MDS). Between several prognostic MDS risk groups, the immune status can be markedly different. Low risk disease is often characterized by an increased number and activation state of pro-inflammatory immune cells (i.e. T helper (Th)17, natural killer (NK)-cells and CD8+ cytotoxic T cells,[1–6)] whereas in high risk disease an immunosuppressive response is the dominant feature (i.e. expansion of T regulatory cells (Tregs,[7–10]) and myeloid-derived suppressor cells [11] which could facilitate immune escape and eventually progression to acute myeloid leukemia (AML). Although an "activated" immune system and associated tumor specific immune responses are crucial for an effective immune surveillance and elimination of the malignant clone, in longer term however, chronic immune stimulation may enhance the risk of genomic instability and development of MDS/AML.[12] Smoldering inflammation as a result of aberrant activation of inflammatory pathways (e.g. Toll-like receptor (TLR) signaling) can induce malignant transformation and disease progression by causing genotoxic cell stress. Indeed, in low risk MDS elevated levels of several stress-inducing molecules, such as the damage-associated molecular pattern (DAMP) molecules S100A8/A9, are actively secreted from mesenchymal niche cells in the bone marrow (BM) microenvironment, thereby causing niche-induced DNA damage in hematopoietic stem and progenitor cells (HSPCs).[13] High S100A9 levels in MDS BM also result in inflammasome assembly and subsequent initiation of pyroptosis, an immunogenic form of cell death, which could potentially explain the high rate of cell death in low risk MDS BM.[14–16] These soluble inflammatory molecules are able to bind to TLRs on the surface of HSPC and immune cells. Constitutively activated TLR-signaling and downstream mitogen-activated protein kinase (MAPK) and NF-κB activation are evident and have been implicated in the pathogenesis of MDS.[17–24] Besides active secretion of stress-inducing molecules, passive release from cells undergoing immunogenic cell death has also been described in MDS. Levels of high mobility group box 1 (HMGB1), a mediator strongly involved in inflammatory processes and a ligand for TLR4, were found to be increased in the BM of MDS patients due to impaired clearance of apoptotic cells causing secondary necrosis and leakage of this molecule into the BM environment.[25]

As a result of this vicious circle of inflammation and cell death, immune-inhibitory mechanisms that interfere with this excessive inflammatory process kick in. While these immune-inhibitory pathways may control the inflammatory response to some extent, they also facilitate the expansion of immunosuppressive cells such as Tregs and MDSCs which further suppress the already weakened

immune-surveillance against the malignant clone. Therefore, a delicate balance between immune activation and inhibition is required to maintain an effective immunosurveillance. Thrombomodulin (TM) is known for its anti-coagulant function by serving as a co-factor for thrombin. Notably, the lectin-like domain of the TM molecule harbors high anti-inflammatory activities and interferes with the complement pathway.[26–28] Several studies have shown strong correlations between disease severity and TM levels in, for instance, autoimmune and infectious diseases as well as in cancer.[29–31] In the immune system, TM, also known as CD141 or BDCA-3, is mainly expressed on dendritic cells.[32–34] We have previously described elevated expression of TM/BDCA-3 on tumor-conditioned and immunosuppressive monocyte-derived dendritic cells that acquire a M2-like macrophage phenotype.[35,36] The anti-inflammatory potential of TM has also been assigned to the fact that TM is able to bind HMGB1, thereby inhibiting the high pro-inflammatory function of this molecule. Since high levels of this molecule were found in low risk MDS BM, this interactive mechanism may be relevant in keeping excessive immune activation to a minimum. The aim of this study was to evaluate the possible role and prognostic value of TM in regulating the inflammatory immune response in MDS. The expression of TM was evaluated on different monocyte subsets (classical, intermediate and non-classical) in the peripheral blood (PB) and BM within different MDS risk groups. Multidimensional mass cytometry (CyTOF) was used to investigate the putative impact of TM+ monocytes on T cell phenotype. Both in the PB as well as in the BM of MDS patients, classical monocytes showed high expression of TM on their cell surface compared to healthy donor-derived monocytes. Expression of TM was related to a more favorable prognosis and functional skewing of the T cell response to a more tolerized state.

Methods

Patient and control samples

Twenty-nine peripheral blood (PB) and 154 bone marrow (BM) samples of newly diagnosed MDS patients were collected in this study. Patients were assigned to different risk categories using the Revised International Prognostic Scoring System (IPSS-R) and the 2016 World Health Organization (WHO) classification (details are given in the supplementary methods file and Table 1). A set of 25 age-matched control BM samples was obtained after written informed consent from hematologically healthy patients that were undergoing cardiac surgery at the Amsterdam UMC. For PB analysis 31

control samples were collected. The study was approved by the local ethical committee and in accordance with the declaration of Helsinki.

Flow cytometry and fluorescence in situ hybridization (FISH)

PB and BM cells were analyzed on a flow cytometer (FACSCanto™, BD Biosciences) after incubation with a panel of monoclonal antibodies (see supplementary methods for details). Data analysis was performed using FlowJo software (Tree star, Ashland, OR, USA). Monocyte subsets were identified based on the differential expression of CD14, CD16 and M-DC8 (anti-6-Sulfo LacNAc [Slan]), using recent recommendations (Figure S1).[37,38] Classical monocytes were characterized by high CD14 expression, and CD16 and M-DC8 negativity. Intermediate and non-classical monocytes were both defined as CD16 positive. However, only intermediate monocytes expressed CD14. We used M-DC8 as a marker to discriminate between intermediate and non-classical monocytes as suggested by Hofer et al (Figure 1A).[38]

Three samples containing monocytes with high TM expression and a known cytogenetic aberrancy were used for the isolation of classical monocytes and subsequent interphase FISH analysis (details are given in supplementary methods).

T cell cultures and multidimensional mass cytometry

A multi-parameter deep-phenotyping strategy, known as cytometry by time-of-flight (CyTOF), was used for T cells cultured in the presence of MDS-derived TM- or TM+ monocytes (culture details can be found in the supplementary methods). Data was analyzed using a combination of automated dimension reduction and clustering methods including t-distributed stochastic neighbor embedding (t-SNE)[39] to visually (viSNE) identify cell populations.[40] This was followed by spanning-tree progression analysis of density-normalized events (SPADE)[41] for the clustering of T cells as published before.[42,43] The deep immunophenotyping of T cell clusters was performed using our in-house pipeline (publicly available here: https://github.com/kordastilab/cytoClustR) followed by Marker Enrichment Modelling (MEM) to calculate MEM scores of the identified subpopulations.[44]

Statistical analysis

Significant differences for two-group comparisons were analyzed by applying a non-parametric Mann-Whitney U test, whereas for multi-group comparisons a Kruskal-Wallis with Dunn's multiple

comparisons test was used. A Spearman correlation was computed in peripheral blood and bone marrow comparisons. P-values of <0.05 were considered significant. Graphpad Prism 6 software (San Diego, USA) was used for graphic display and statistical calculations. A multivariate Cox regression analysis for overall and leukemia-free survival was performed by using the IBM SPSS Statistics software version 22 (New York, USA).

Results

Classical monocytes express thrombomodulin in MDS patients

Monocyte subsets were identified by flow cytometric analysis based on the expression of CD14, CD16 and M-DC8 according to recently published recommendation.[38] Classical, intermediate and non-classical monocytes were characterized by using the above mentioned markers (Figure 1A). Then, the expression of TM and HLA-DR, a major histocompatibility complex (MHC) molecule class II, was assessed on all monocyte subsets derived from normal bone marrow (NBM) and MDS BM samples. Monocyte subsets from MDS patients showed high levels of TM expression on their cell surface, whereas NBM-derived monocytes showed very low levels of TM. Also, HLA-DR expression was higher on all monocyte subsets in MDS BM than it was in NBM (Figure 1A). Quantification of these expression levels on different monocyte subsets was performed in a larger cohort of patients (n=10 NBM and n=24 MDS BM samples). Total percentages of monocyte subsets in MDS BM compared to NBM were not significantly different. However, the percentage of monocytes that expressed TM was significantly higher for MDS-derived classical monocytes compared to the same monocyte subset in NBM (37.3% *vs* 9.9%, p<0.0001, Figure 1B). The percentage of TM expression on intermediate and non-classical monocytes was equally distributed between MDS BM and NBM (Figure 1B). The MFI of TM and HLA-DR was evaluated on the three distinct monocyte subsets using the same set of samples. Similarly, classical monocytes from MDS BM showed higher expression levels of TM compared to NBM classical monocytes (3.7-fold, p<0.0001). HLA-DR expression levels were higher for all MDS-derived monocyte subsets (classical monocytes: 2.0-fold, p=0.0015; intermediate monocytes: 1.8-fold, p=0.0154; non-classical monocytes: 1.5-fold, p<0.0001, Figure 1B). TM expression remained unchanged upon overnight stimulation with TLR-ligands in a preliminary set of samples (Figure S2).

Classical monocytes were then analyzed in a larger set of samples since this subset forms the most prevalent subset in NBM as well as in MDS BM and it revealed the most prominent difference in TM

expression. Also, the peripheral blood (PB) compartment was included in the analysis. The cohort was extended with 130 MDS BM-derived samples and 15 NBM samples. The PB and BM samples in the extended control and patient cohort (n=31 normal PB (NPB), n=29 MDS PB, n=25 NBM and n=154 MDS BM) were screened for the presence of TM on classical monocytes (Figure 1C). MDS-derived classical monocytes showed elevated expression of TM in both the PB as well as in the BM compartment compared to NPB and NBM samples (PB: 33.6% *vs* 17.8%, p=0.015; BM: 37.0% *vs* 8.6%, p<0.0001). Furthermore, a strong positive correlation was found for the percentage of classical monocytes expressing TM in the two compartments in 25 paired MDS samples (r=0.83, p<0.0001. Figure 1C). In order to study TM expression on other cell types present in the PB and BM compartment, the same flow cytometric panel was used. We were able to identify granulocytes, eosinophils and B cells. TM was exclusively expressed on MDS-derived monocytes (Figure S3) and none of the other cell types in PB and BM showed positivity for TM, including the non-B cell lymphocytic compartment consisting of T and NK cells (data not shown).

TM-expressing monocytes are clonally involved and are associated with low risk MDS features

In order to investigate the clonal involvement of TM positive monocytes in MDS, cells from three different patients were sorted and screened for the presence of a known cytogenetic aberration according to their karyotype. Almost all monocytes of these patients showed high expression of TM.

One patient had a deletion of chromosome 5q (del5q) in all cells (karyotype: 46,XY,del(5)(q22q33)[10]), one showed trisomy for chromosome 8 in 65% of the cells (karyotype: 47,XY,+8[13]/46,XY[7]) and one case had a monosomy 7 (karyotype: 45,XY,-7[10]). In all cases, TM+ monocytes were highly involved in the dysplastic clone and showed a high percentage of cells with the respective cytogenetic abnormality, suggesting that they do not come from healthy CD34+ cells. The isolated CD34+ progenitor cells showed a similar pattern, since the known cytogenetic aberration was present in most analyzed cells. As expected, B cells were not involved. Partial involvement was found for whole bone marrow samples (Figure 2A).

To further correlate TM expression with different MDS subgroups, clinical patient data were collected and the IPSS-R, reflecting survival and the risk for disease progression, was calculated. Furthermore, patients were categorized by using the 2016 WHO classification system that incorporates clinical characteristics, peripheral blood and bone marrow findings and cytogenetic analysis. The percentage of monocytes displaying TM expression was higher for patients that had a very low or low risk score in

the IPSS-R as compared to patients in higher risk groups and healthy controls (very low/low 40.1% *vs* intermediate 22.7% *vs* high/very high 28.3% *vs* NBM 11.3%. Figure 2B). Additionally, TM expression was elevated in WHO categories related to lower risk disease such as MDS-SLD and MDS-MLD with or without ring sideroblasts (RS) compared to categories related to higher risk MDS, i.e. EB-1 and EB-2. Compared to NBM, the percentage of monocytes expressing TM was higher in all WHO subgroups (SLD/MLD/RS-SLD/RS-MLD 40.9% *vs* EB-1/EB-2 24.2% *vs* NBM 11.3%. Figure 2B). Using the percentage of BM blast cells as a reflection of disease stage, patients with blast percentages below 5% harbored higher numbers of TM+ monocytes than patients with 5% or more blast cells (41.3% vs 25.7%, respectively, $p<0.0001$. Figure 2C). Finally, a relation between the percentage of monocytes expressing TM and the presence of ring sideroblasts (erythroblasts with mitochondrial iron accumulation) was found (present 45.4% *vs* absent 33.2%, $p=0.003$. Figure 2C). As an "indirect" indication of the presence of a *SF3B1* mutation, the percentage of TM+ monocytes was compared between RS subtypes and other MDS subtypes (Figure S4). Significant higher percentages of TM+ monocytes in RS cases was found compared to EB-I/II. A trend of higher frequencies was observed for the comparisons with other subtypes.

MDS-derived TM+ monocytes polarize CD4+ T cells to an immunosuppressive phenotype

The next research question was to study the effect of TM+ monocytes on the phenotype of CD4+ T cells and whether they could induce an anti-inflammatory T cell phenotype. Healthy donor-derived CD4+ T cells were co-cultured with sorted TM- or TM+ monocytes from two MDS patients (Figure S5). After 5 days, T cells were harvested and labelled with a comprehensive panel of metal tagged antibodies (Table S1) for mass cytometry (CyTOF). Using our data analysis pipeline, T cell subsets were identified (Figure S6) and compared between the conditions (i.e. day zero, control stimulated and cultures with TM- or TM+ monocytes. Figure 3A). Compared to day zero, all conditions showed expansion of specific T cell subsets. To further characterize these cell islands, T cells were clustered by SPADE on tSNE (see materials and methods). Using selected T cell markers various subsets could be characterized (Figure 3B). Then frequencies of clusters between the conditions were compared (Figure 3C). Nodes highlighted with red circles refer to the most frequent T cell clusters in the TM- condition whereas black circles indicate a higher percentage in the TM+ condition. For both conditions the top 5 of highest frequencies was selected. The expression profiles of T cells in the identified clusters were evaluated next, using MEM (see materials and methods). T cell clusters were divided

into two groups for comparison: clusters with highest frequency in the TM+ condition (called “up”) and clusters with highest frequency in the TM- condition (called “down”). MEM scores were calculated for each marker in each group for further comparison.[44] Interestingly, T cells that are predominantly present upon culturing with TM+ monocytes (group called “up”) show an anti-inflammatory profile (Figure 3C). They are polarized toward Th2, Treg and PD-1 expressing clusters of T cells, since they express high levels of FoxP3, GATA3 and CD279 (PD-1) and have elevated concentrations of intracellularly measured IL-4 and IL-10. In contrast, T cells cultured in the presence of TM- monocytes (group called “down”) are mainly positive for IFN-y and hardly for immunosuppressive cytokines.

The presence of TM+ monocytes is related to a better overall and leukemia-free survival

In order to determine the clinical significance of the presence of TM+ monocytes the overall survival (OS) and leukemia-free survival (LFS) were calculated. As a cut-off for TM expression monocytes from the healthy donor cohort were used. The mean TM percentage plus 2SD was calculated resulting in a cut-off of 25.53%. In total, OS data for 122 MDS patients and LFS data for 102 patients was available. Interestingly, the presence of TM on MDS monocytes was significantly associated with a better OS ($p=0.006$) as well as a better LFS ($p=0.029$. Figure 4A). The median OS for patients with TM+ monocytes was 58 months, whereas the median OS without TM+ monocytes was 30 months. For a subgroup of patients, data were available for further risk stratification into IPSS and IPSS-R risk groups. Following the hypothesis that TM is mainly present in an inflammatory environment, survival and LFS data was also analyzed in low risk MDS groups (i.e. IPSS low and intermediate groups or IPSS-R very low, low and intermediate groups). Similarly, the presence of TM+ monocytes in this subgroup of patients resulted in a better overall survival (Figure 4B). Probably because of low patient numbers a difference in LFS didn’t reach significance (Figure 4C). To test if TM as a single marker has an independent prognostic value in overall and leukemia-free survival a multivariate Cox regression analysis with backward stepwise elimination was performed. Covariates that were included in this analysis included hemoglobin levels, absolute neutrophil count, platelet count, bone marrow blast percentage, cytogenetic risk group and percentage of TM expression on monocytes. For 60 patients information on all variables was available (not shown). Covariates with a p-value of >0.10 were removed. Both the cytogenetic risk group as well as the percentage of TM expression were predictive markers for OS ($p=0.001$ and $p=0.064$ respectively). For LFS the percentage of blast and TM expression had predictive value ($p=0.010$ and $p=0.077$ respectively).

Discussion

The BM microenvironment in MDS, and particularly in low risk groups, is often characterized by the presence of pro-inflammatory cells and molecules. While an increased inflammation and subsequent cellular immune response is crucial to eliminate malignant cells, the continuous immune stimulation could lead to genomic instability and inevitable malignant transformation.[12] Identifying the factors that could maintain a healthy overall immune response in MDS is important and clinically relevant. In mice, it has been shown that lack of the lectin-like domain of TM leads to reduced survival after endotoxin exposure, whereas a recombinant form diminished NF-κB and MAPK activation.[45] In the current study, we therefore investigated the presence of TM on immune cells in MDS-derived BM and PB samples and its immune-modulatory role in MDS. TM was mainly expressed on monocyte subsets and not on granulocytes, lymphocytes or eosinophils. The difference in TM expression between monocytes of healthy donors and MDS was most evident for classical monocytes. FISH analysis showed clonal involvement for these TM+ monocytes. This subset showed hardly any TM expression on either healthy BM- or healthy PB-derived cells. In MDS patients there was a high variation in monocytes expressing TM. Some patients showed expression levels similar to healthy donor-derived monocytes, whereas for others nearly all monocytes expressed TM. We found that the percentage of TM expression was evidently higher in low risk groups as compared to higher risk categories, suggesting that the presence of TM may be primarily observed in a pro-inflammatory environment (i.e. low risk MDS). Interestingly, Talati et al previously described a correlation between the presence of classical monocytosis in MDS and favorable prognostic factors such as increased white blood cell counts and absolute neutrophil counts.[46] Furthermore, an increased percentage of monocytes was associated with lower MDS risk groups and good-risk cytogenetics. SF3B1 was present in greater frequency in this MDS group and overall survival tended to be better for these patients. In this perspective, it would be interesting to investigate the presence of TM in a same set of samples in future research.

Besides active secretion and passive release of inflammatory molecules in the MDS BM environment, certain T cell subsets, particularly Th1 and Th17 type T cells, contribute to an immune-active state by the secretion of high amounts of interferon (IFN)-γ and IL-17. In order to investigate the effect of monocytes on T cell skewing, healthy T cells were cultured in the presence of TM+ or TM- MDS monocytes. Using mass cytometry, we were able to utilize a comprehensive panel of surface and intracellular markers for this purpose. Interestingly, T cells cultured in the presence of TM+ monocytes

showed an anti-inflammatory skewed profile. They showed less IFN-γ positivity and higher concentrations of IL-4 and IL-10 were measured intracellularly compared to T cells cultured with TM- monocytes. While the number of patients' samples are limited and data need to be interpreted with caution, these data suggest that TM+ monocytes polarize T cells toward Th2 and/or Treg phenotypes. Altogether, our data point to an interesting function for TM-expressing monocytes in the highly inflammatory environment of low risk MDS patients. They could play an essential role in dampening disproportionate immune activation by inducing anti-inflammatory T cell subsets. In keeping with this notion, overall and leukemia-free survival was better for patients in which the BM contained TM+ monocytes than for patients lacking TM expression, supporting a clinically relevant mechanism. Nevertheless, in longer term, this mechanism could lead to a profound immunosuppressive state which prevent effective immune surveillance; a common condition in higher risk MDS.

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Table 1. Patient and control characteristics

Characteristics	Value	[min-max]
Total #		
HD	56	
MDS	183	
Peripheral blood samples		
Number	60	
HD	31	
MDS	29	
Age – mean, y		
HD	-	
MDS	69	[45-85]
Sex		
HD – male/female	-	
MDS – male/female	20/9	
Bone marrow samples		
Number	179	
HD	25	
MDS	154	
Age – mean,y		
HD	62	[20-86]
MDS	69	[36-94]
Sex		
HD – male/female	18/7	
MDS – male/female	110/44	
IPSS-R		
Very low risk	20	
Low risk	34	
Intermediate risk	22	
High risk	8	
Very high risk	8	
WHO		
MDS-SLD	5	
MDS-MLD	48	
MDS-RS-SLD	8	
MDS-RS-MLD	27	
MDS-EB1	22	
MDS-EB2	20	
Del5q	12	
% Blasts		
<5%	97	
≥5%	44	
Ring sideroblasts		
No	106	
Yes	40	

Abbreviations: EB, excess blasts; HD, healthy donor; MLD, multilineage dysplasia; RS, ring sideroblasts; SLD, single lineage dysplasia; WHO, World Health Organisation; y, years.

Figure legends

Figure 1. TM-expressing monocyte subsets in normal and MDS bone marrow and peripheral blood samples. (A) Identification of different monocyte subsets in normal bone marrow (NBM) and MDS BM. Three markers, CD14, CD16 and M-DC8, were used to identify classical (CD14++CD16-M-DC8-, in orange), intermediate (CD14+CD16+M-DC8-, in purple) and non-classical monocytes (CD14+/-CD16+M-DC8+, in green). The expression levels of thrombomodulin (TM) and HLA-DR were assessed on all separate monocyte subsets. The median fluorescence intensity (MFI) value for each subset is shown for a representative sample. (B) Frequencies of monocyte subsets in the BM of 10 healthy individuals and 24 MDS patients. Percentages were calculated from the total CD45+ mononuclear cell fraction. Mean frequencies ± SEM are given (NBM *vs* MDS BM: classical monocytes 11.47% SEM ± 1.86 *vs* 11.11% SEM ± 2.20, intermediate monocytes 0.45% SEM ± 0.12 *vs* 0.85% SEM ± 0.17, non-classical monocytes 0.46% SEM ± 0.11 *vs* 0.38% SEM ± 0.05). Furthermore, the percentage of monocytes that express TM is displayed (NBM *vs* MDS BM: classical monocytes 9.94% SEM ± 2.82 *vs* 37.27% SEM ± 4.00, intermediate monocytes 42.60% SEM ± 7.27 *vs* 54.90% SEM ± 3.48, non-classical monocytes 45.11% SEM ± 3.72 *vs* 49.46% SEM ± 3.34). Expression levels of thrombomodulin and HLA-DR on NBM and MDS BM monocyte subsets are also displayed. Mean MFI values ± SEM are shown for 10 NBM and 24 MDS BM samples (TM in NBM *vs* MDS BM: classical monocytes 382 SEM ± 65 *vs* 1425 SEM ± 367, intermediate monocytes 1414 SEM ± 245 *vs* 2208 SEM ± 259, non-classical monocytes 1279 SEM ± 169 *vs* 1676 SEM ± 144. HLA-DR in NBM *vs* MDS BM: classical monocytes 5426 SEM ± 715 *vs* 11010 SEM ± 1056, intermediate monocytes 20062 SEM ± 2529 *vs* 35639 SEM ± 3989, non-classical monocytes 10117 SEM ± 856 *vs* 15255 SEM ± 1915). (C) Percentage of TM-expressing classical monocytes in peripheral blood (PB) and bone marrow (BM). Bars indicate mean frequencies (Normal PB (NPB; n=31) *vs* MDS PB (n=29): 17.8% *vs* 33.6%. Normal BM (NBM; n=25) *vs* MDS BM (n=154): 8.6% *vs* 37.0%). Thrombomodulin expression was correlated in peripheral blood- and bone marrow-derived classical monocytes. In total, 25 paired MDS samples were included. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Figure 2. Classical monocytes are clonally involved and the presence of thrombomodulin correlates with disease states. (A) Sorted cells, including B cells and CD34+ blast cells, from patients with a known cytogenetic aberrancy were subjected to a FISH analysis. Furthermore, whole BM samples were used for degree of cytogenetic load. Representative interphase cells hybridized with

the chromosome 5q probe, showing loss of 5q in CD34+ blasts and monocytes, and no loss of 5q in B-cells are shown. In three tested cases (monosomy 7, del 5q and trisomy 8), classical monocytes and CD34+ blast cells were highly involved in the dysplastic clone, whereas B cells were not involved. Interphase FISH on whole bone marrow samples showed both an aberrant and a normal cell line. (B) TM+ classical monocytes in different risk groups within the IPSS-R and according to the WHO2016 classification. The highest percentage of classical monocytes that express TM is found in the very low-low risk groups, whereas in higher risk groups the percentage of TM expression is significantly reduced compared to low risk disease (NBM (n=25) 11.3% SEM ± 3.0% *vs* very low/low (n=54) 40.1% SEM ± 2.9% *vs* intermediate (n=22) 22.7% SEM ± 3.7% *vs* high/very high (n=16) 28.3% SEM ± 4.9%). Patients having a low risk-related WHO2016 classification (SLD/MLD/RS-SLD/RS-MLD) show higher percentages of TM expression on monocytes compared to higher risk groups (EB-1/EB-2) and NBM. (NBM (n=25) 11.3% SEM ± 3.0% *vs* SLD/MLD/RS-SLD/RS-MLD (n=87) 40.9% SEM ± 2.3% *vs* EB-1/EB-2 (n=42) 24.2% SEM ± 2.7%). (C) The percentage of classical monocytes that is positive for TM in patients with low and higher blast counts. In the group of patients that have blast counts below 5%, the percentage of monocytes expressing TM is significantly higher compared to the group of patients with blast counts ≥5% (<5% (n=97) 41.3% SEM ± 2.2% *vs* ≥5% (n=44) 25.7% SEM ± 2.8%). Patients with ring sideroblasts show higher percentages of monocytes positive for TM than patients that do not have ring sideroblasts present (Yes (n=40) 45.4% SEM ± 3.7% *vs* No (n=106) 33.2% SEM ± 2.0%). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Abbreviations: EB, excess blasts; IPSS-R, Revised International Prognostic Scoring System; MLD, multilineage dysplasia; NBM, normal bone marrow; RS-MLD, ringed sideroblasts with multilineage dysplasia; RS-SLD, ringed sideroblasts with single lineage dysplasia; SLD, single lineage dysplasia.

Figure 3. Deep phenotyping of T cells after co-culture with TM- or TM+ MDS monocytes. Healthy donor-derived CD4+ T cells were co-cultured with MDS TM- or TM+ monocytes (from 2 MDS patients), or in the stimulated control condition with plate-bounded anti-CD3 antibody only. After a 5-days culture, T cells were stained with an extensive panel of surface and intracellular markers as well as transcription factors and cytokines and analyzed using mass cytometry (CyTOF). (A) Viable T cells were identified and visualized using viSNE. Different T cells subsets were identified based on IFN-γ, GATA3, IL-17, IL-4, FoxP3 and CD127 (Th1 were considered to be IFN-y+, IL-17 and GATA3-; Th2 were GATA3+IL-4+; Th17 were IL-17+; Tregs were CD127- and FoxP3+CD25+ and IFN-γ-. Figure

S6). Overlays were created from condition-specific biaxial viSNE contour plots and each individual T cell subset. T cell populations at start of the experiment (D=0) and after 5 days of culture with anti-CD3 only, TM- monocytes or TM+ monocytes are shown for a representative sample. (B) Cells were then further clustered with SPADE into 50 nodes using the clustering channels tSNE1 and tSNE2. Different clusters of nodes representing various T cell subsets could be identified in the stimulated control condition by using selected markers. The color intensity in each node reflects the expression level of the indicated marker and the size of the node reveals the number of cells involved. (C) Frequencies of all clusters were compared between the TM- and TM+ conditions. T cell clusters that were most prevalent in the TM- condition are highlighted in red. Black circles represent clusters that show higher percentages in the TM+ condition. The top 5 of highest frequencies for both conditions is shown. Using MEM, profiles of T cell clusters with highest frequencies in TM- or TM+ cells were characterized. Two subgroups of clusters were generated: 1) five nodes with highest frequency in TM+ condition (called “up”), 2) five nodes with highest frequency in TM- condition (called “down”). Expression levels of given markers are shown for the TM+ as well as the TM- conditions, showing that global expression in the identified set of clusters for the different markers is nearly similar in both conditions. MEM scores were calculated for the markers and results are presented in a heat map. The group of nodes that are present in a higher percentage in cultures with TM+ monocytes compared to cultures with TM- monocytes reflect an anti-inflammatory profile. T cells in this group (all within “non-Treg nodes”) express higher levels of FoxP3, GATA3, CD279 (PD-1), IL-4 and IL-10, a phenotype which suggests they are polarizing toward Treg phenotype. Abbreviations: Ir, iridium; MEM, Marker Enrichment Modelling; Rh, rhodium; SPADE, spanning-tree progression analysis of density-normalized events; TM, thrombomodulin; tSNE, t-Distributed Stochastic Neighbor Embedding.

Figure 4. Overall and leukemia-free survival is related to the presence of TM+ monocytes. As a cut-off percentage for the presence of TM on MDS monocytes, expression rates in the healthy donor cohort was used. The mean percentage + 2SD was calculated resulting in a cut-off of 25.53%. Statistical differences were calculated using the log-rank test. (A) Overall survival data for 122 MDS patients and leukemia-free survival data for 102 patients. A significant difference in overall survival was found between the presence and absence of TM on monocytes in MDS BM (p= 0.006). The median overall survival for patients with TM+ monocytes was 58 months and for patients without TM 30 months. The time to development of leukemia was significantly longer for patients with TM

expression compared to patients without TM expression (p= 0.029). (B) Patients were further selected based on their low risk status. Survival curves are shown for the presence and absence of TM+ monocytes in low risk patients according to the IPSS and IPSS-R. (C) Additionally, the leukemia-free survival for low risk patients is shown in the TM+ and TM- group. Abbreviations: IPSS, International Prognostic Scoring System; IPSS-R, Revised International Prognostic Scoring System; LFS, leukemia-free survival; TM, thrombomodulin.

Figure 1

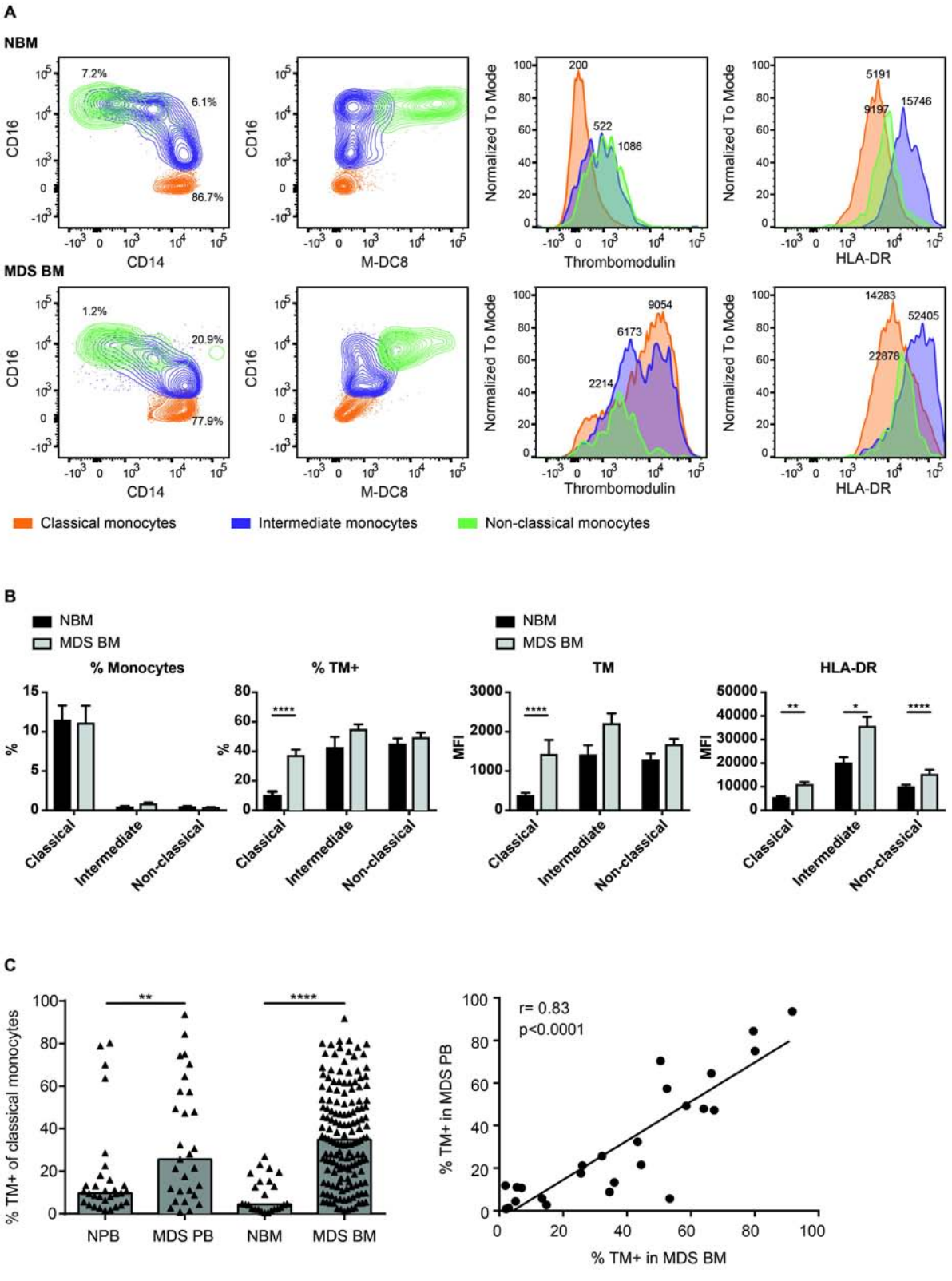
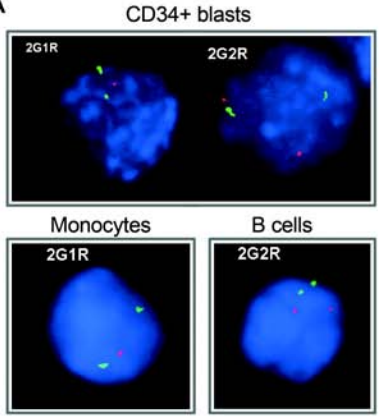

A
NBM
7.2%
6.1%
86.7%
CD16
CD14
M-DC8
Normalized To Mode
200
522
1086
Thrombomodulin
5191
9197
15746
HLA-DR
MDS BM
1.2%
20.9%
77.9%
9054
6173
2214
14283
52405
22878
Classical monocytes
Intermediate monocytes
Non-classical monocytes
B
NBM
MDS BM
% Monocytes
% TM+
TM
HLA-DR
%
MFI
****
**
*
Classical
Intermediate
Non-classical
C
% TM+ of classical monocytes
NPB
MDS PB
NBM
MDS BM
r= 0.83
p<0.0001
% TM+ in MDS PB
% TM+ in MDS BM

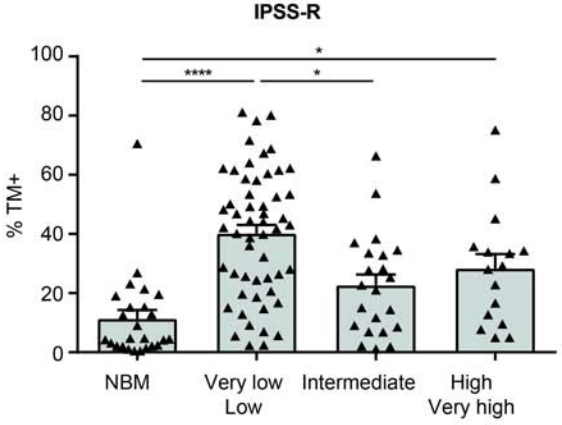
Figure 2

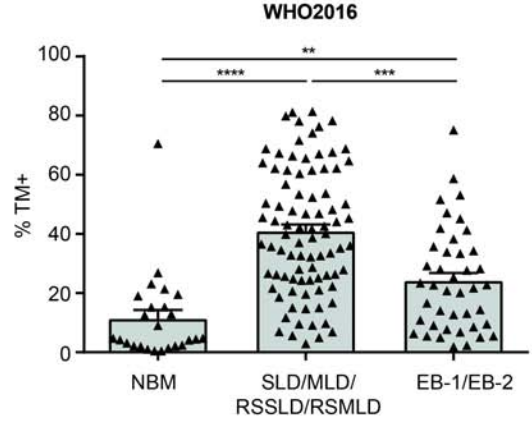
A



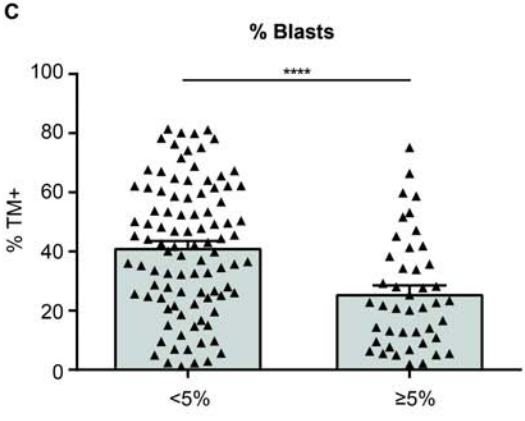
Cell type	FISH results: number of cells	
	Monosomy 7	**Two chromosome 7**
Whole bone marrow	57	43
Monocytes	99	1
B cells	0	100
CD34+ blasts	96	4
	Del 5q	**Normal chromosome 5**
Whole bone marrow	163	37
Monocytes	93	7
B cells	2	98
CD34+ blasts	96	4
	Trisomy 8	**Two chromosome 8**
Whole bone marrow	56	44
Monocytes	81	19
B cells	0	91
CD34+ blasts	77	23

B





C



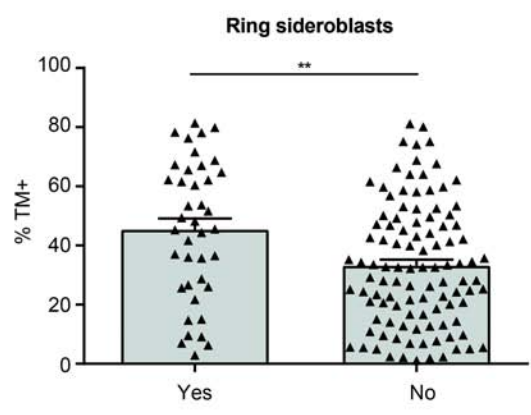
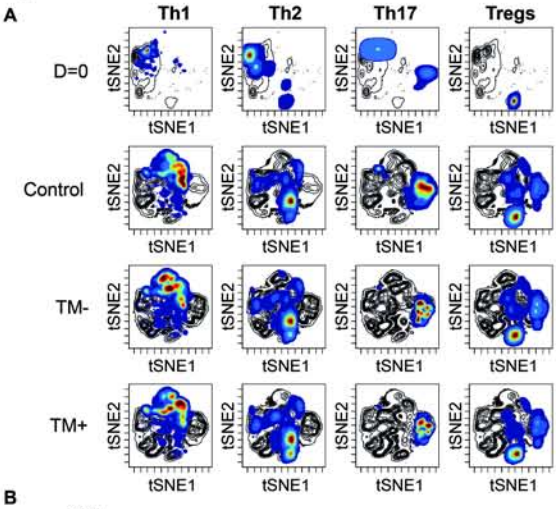
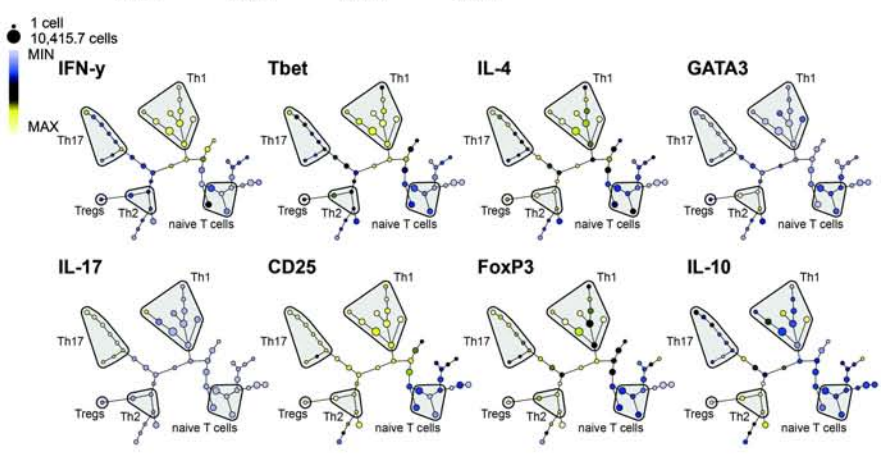


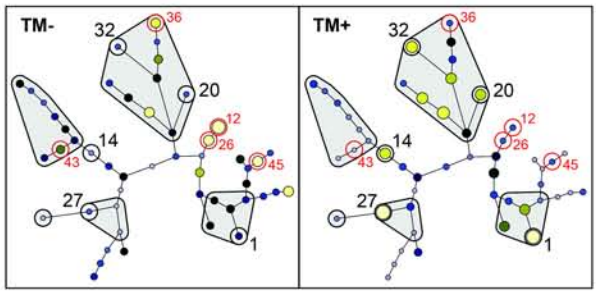
Figure 3





C

Node	TM-	TM+
27	1.12	4.03
1	2.00	4.51
32	1.09	3.44
14	0.78	3.10
20	0.96	3.04
12	3.99	0.74
26	3.99	1.27
36	3.12	1.03
45	3.08	1.20
43	2.00	0.28



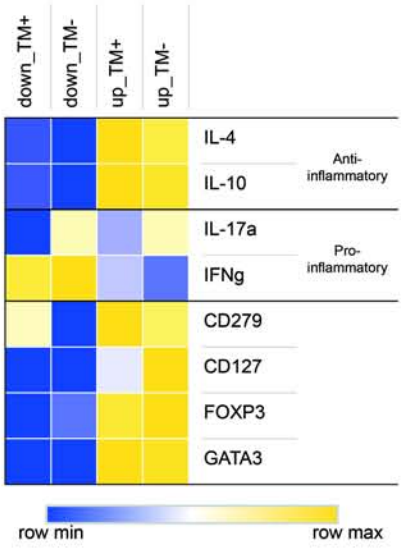
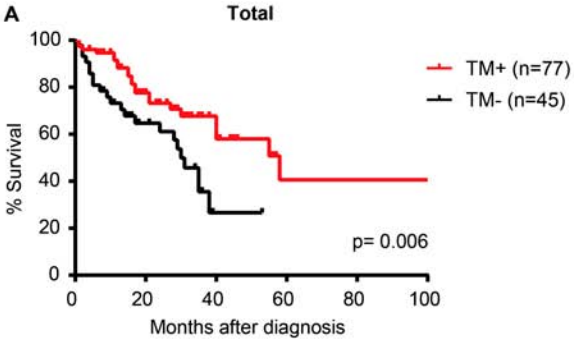
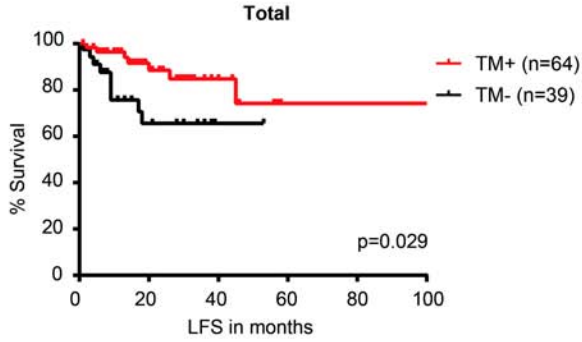
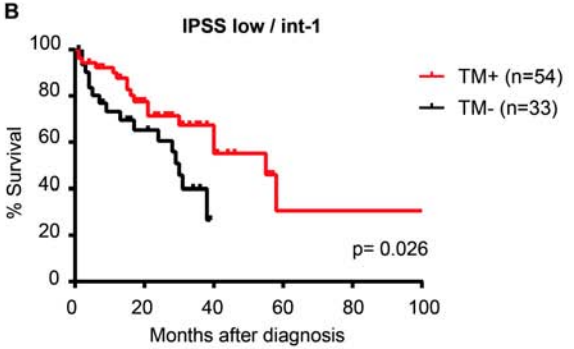
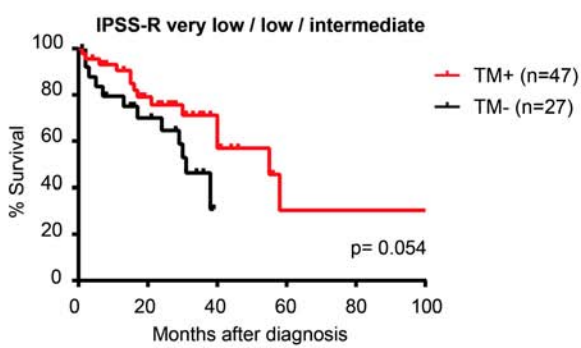


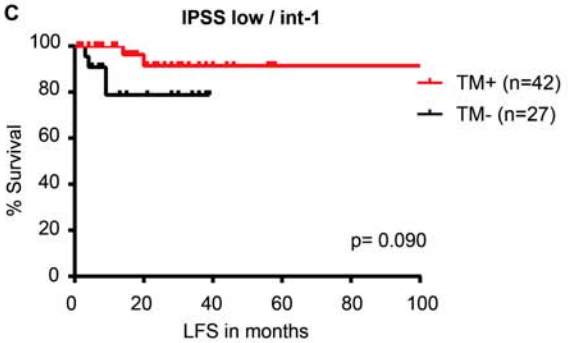
Figure 4

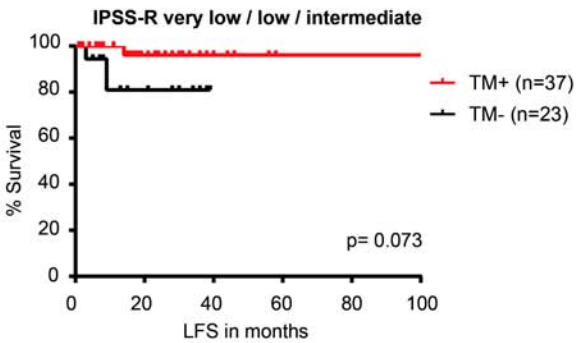

A
Total
% Survival
TM+ (n=77)
TM- (n=45)
p= 0.006
Months after diagnosis


Total
% Survival
TM+ (n=64)
TM- (n=39)
p=0.029
LFS in months


B
IPSS low / int-1
% Survival
TM+ (n=54)
TM- (n=33)
p= 0.026
Months after diagnosis


IPSS-R very low / low / intermediate
% Survival
TM+ (n=47)
TM- (n=27)
p= 0.054
Months after diagnosis


C
IPSS low / int-1
% Survival
TM+ (n=42)
TM- (n=27)
p= 0.090
LFS in months


IPSS-R very low / low / intermediate
% Survival
TM+ (n=37)
TM- (n=23)
p= 0.073
LFS in months

Supplementary methods

Patient and control samples

IPSS-R risk groups of 92 patients could be calculated. In total, 20 patients were considered very low risk, 34 low risk, 22 intermediate, 8 high and 8 very high risk. The 2016 World Health Organization (WHO) classification was available for 142 patients. Five patients could be classified as MDS with single lineage dysplasia (MDS-SLD), 48 as MDS with multilineage dysplasia (MDS-MLD), 8 as MDS with ringed sideroblasts with single lineage dysplasia (MDS-RS-SLD), 27 as MDS with ringed sideroblasts with multilineage dysplasia (MDS-RS-MLD), 22 as MDS with excess blasts-1 (MDS-EB-1) and 20 patients had MDS with excess blasts-2 (MDS-EB-2). Furthermore, 12 patients were assigned to the group involving isolated deletion of chromosome 5q (Table 1).

Flow cytometric analysis was performed on total white blood cells after erythrocyte lysis. Furthermore, BM samples were centrifuged on high speed programs for the collection of platelet-poor BM-derived plasma. These samples were stored at -30°C until they were used for cytokine analyses.

Flow cytometry

PB and BM cells were analyzed on a flow cytometer (FACSCanto™, BD Biosciences) after incubation with a panel of monoclonal antibodies (mAb) consisting of M-DC8-FITC and CD303-FITC (both Miltenyi Biotec, Utrecht, The Netherlands), CD16-PE (Beckman Coulter, Brea, USA), CD11c-PerCP-Cy5.5 (BD Biosciences), CD1c-Pe-Cy7 (eBioscience, San Diego, USA), CD141-APC (Miltenyi Biotec), CD14/CD19-APC-H7, HLA-DR-V450 and CD45-KO (all, BD Biosciences).

Frequencies of classical, intermediate and non-classical monocytes were calculated as percentage of CD45+ mononuclear cells (MNC). Median fluorescence intensity (MFI) levels of TM (CD141/BDCA-3) and HLA-DR were measured on all three subsets and percentages of monocytes positive for TM were calculated as percentage of total monocytes as well as of CD45+ MNC. Furthermore, TM expression on MDS-derived monocytes was evaluated after overnight stimulation and compared to TM expression at baseline (Figure S2). For further functional assays, only stimulated monocytes were used and no unstimulated condition was included (also because of limited cell numbers). Without stimulation co-stimulatory molecules, mainly CD80, were not up-regulated as compared to stimulated conditions (Figure S2).

Fluorescence in situ hybridization (FISH)

Frozen vials with MNC were rapidly thawed and stained with a monoclonal antibody cocktail containing M-DC8, CD1c, CD11c, CD14, CD19, CD34, CD45, CD141 and HLA-DR. TM+ monocytes, CD34+ myeloid progenitor cells and CD19+ B cells were flow cytometrically sorted (BD FACSAria^{TM}). Cells were collected in small Eppendorf tubes and further processed for interphase FISH analysis. They were fixed with 3:1 methanol/acetic acid and transferred to a microscopic slide. FISH was performed on each sorted cell sample according to the manufacturer's protocol using probes LSI EGR1(5q31)/D5S23,D5S721(5p15.2) Dual Colour Probe Set, LSI D7S486(7q31)/CEP7, and LSI CEP8 (D8Z2) (all probes from Abbott Molecular, Des Plaines, IL). For each probe at least 100 cells were investigated. In samples with less than 100 cells on the slide, all cells present were evaluated.

T cell cultures and multidimensional mass cytometry

TM- and TM+ monocytes from fresh samples of two MDS patients (MDS-RS-MLD) were flow cytometrically sorted (Figure S4). They were incubated and stimulated overnight with the TLR-4 ligand LPS (100 ng/ml, Sigma-Aldrich, St. Louis, USA). Next, total CD4+ T cells were derived from a healthy donor by magnetic isolation (Miltenyi Biotec, Utrecht, The Netherlands). Thereafter, T cells were co-cultured with either TM- or TM+ MDS monocytes on a plate pre-coated with anti-CD3 in a ratio of 1:10 for 5 days. T cells from day zero, cultured in the presence of monocytes, or cultured alone were stained with an extensive antibody panel containing cell surface markers, transcription factors and cytokines. Each antibody was tagged to a metal isotope (Table S1) and data was acquired on a Helios mass cytometer (Fluidigm).

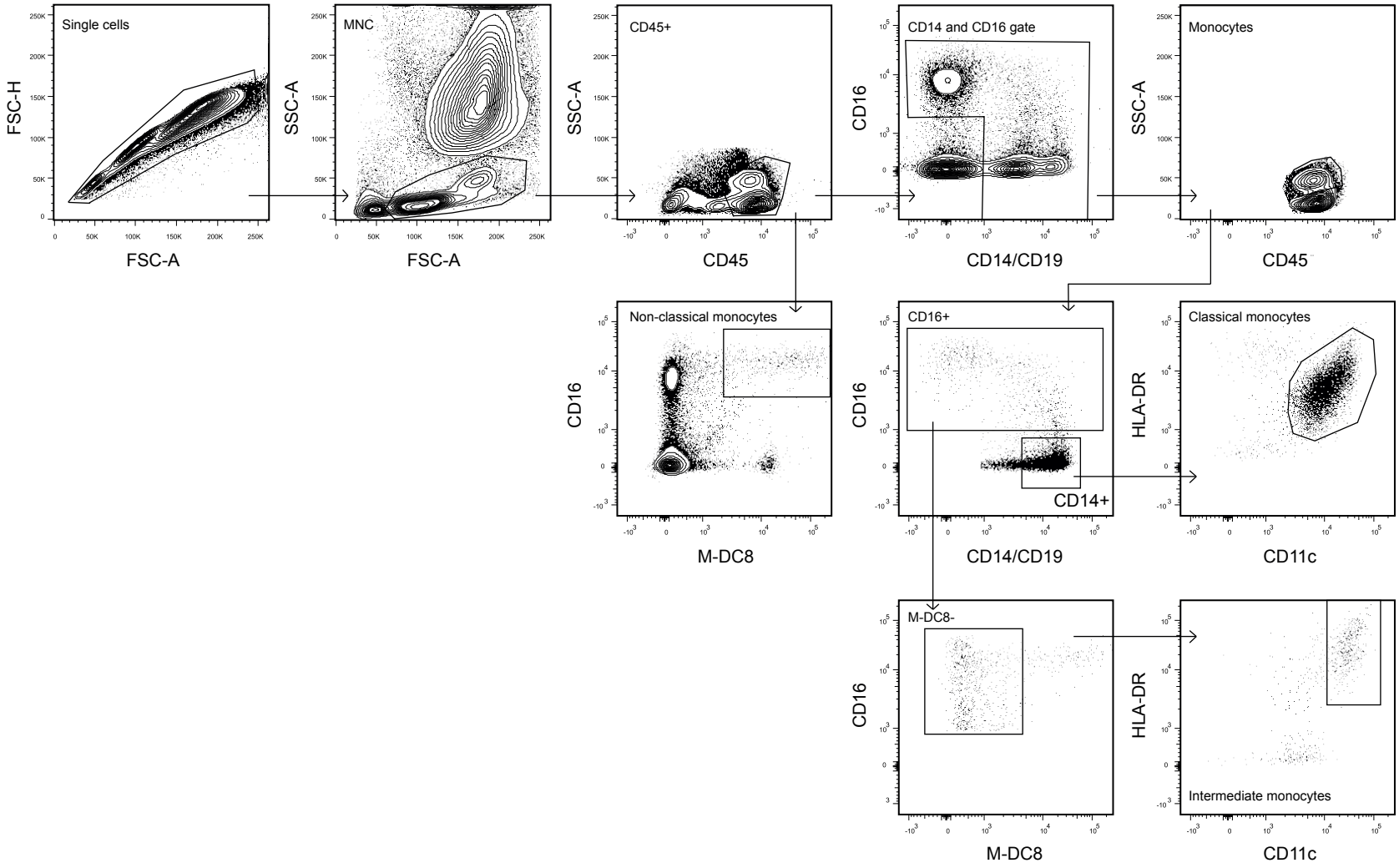


Figure S1. Gating strategy for different monocyte subsets. First, doublets were removed and the CD45+ mononuclear cell (MNC) fraction was selected. Monocytes were gated based on CD16/CD14 and CD45/SSC characteristics. Lymphocytes were removed. Then classical monocytes were identified using CD11c, HLA-DR and high expression of CD14. Intermediate monocytes were gated by using CD16, negative to intermediate expression of CD14 and negativity for M-DC8. The population was cleaned up by a HLA-DR/CD11c gate. CD16/M-DC8 was used for the identification of non-classical monocytes directly from the CD45+ MNC.

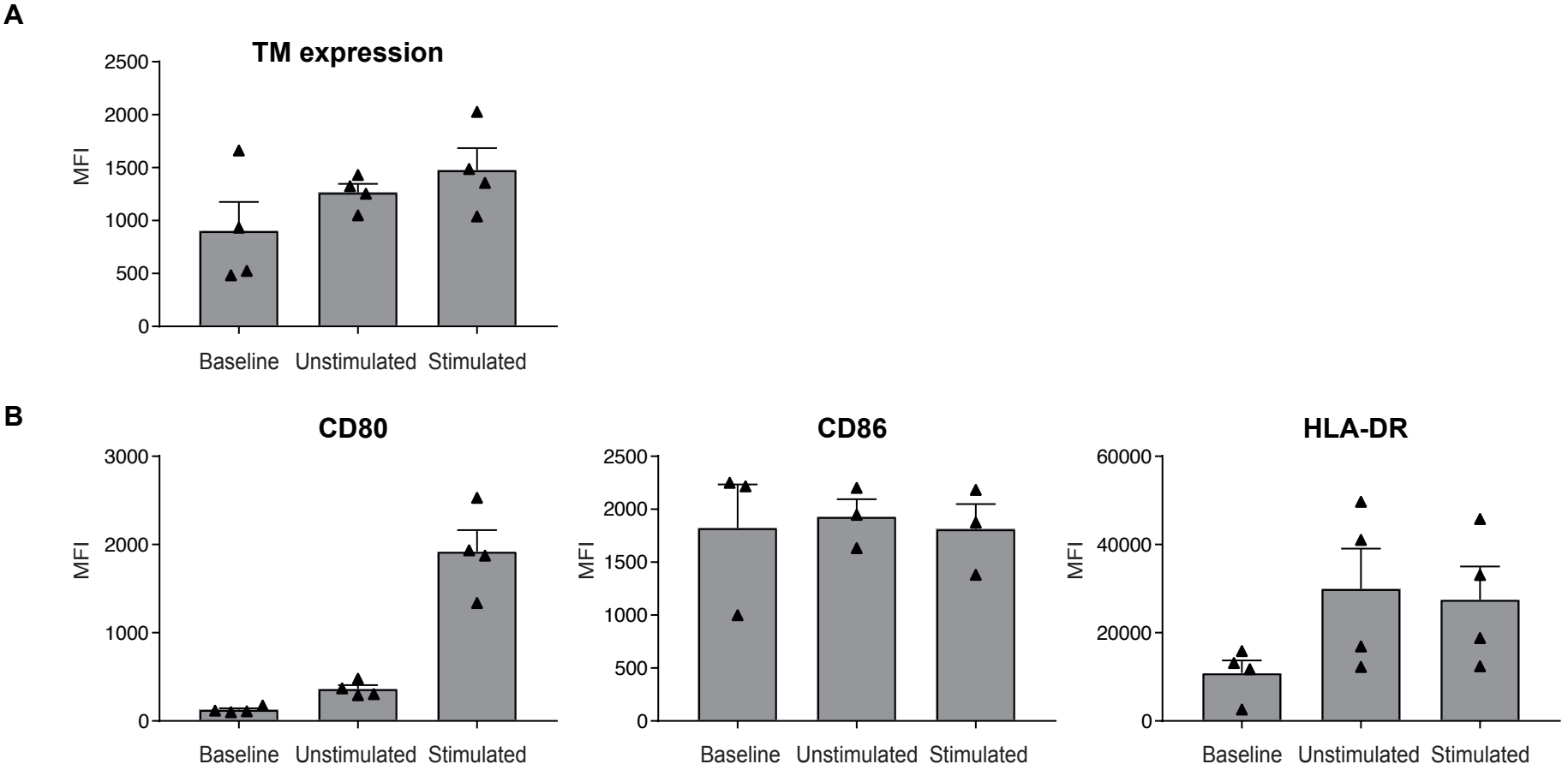


Figure S2. (A) Expression of thrombomodulin on MDS-derived monocytes at baseline and after overnight culture in which monocytes were either left unstimulated or were stimulated with TLR-ligands. (B) Expression of the activation markers CD80, CD86 and HLA-DR on MDS-derived monocytes upon stimulation and without stimulation.

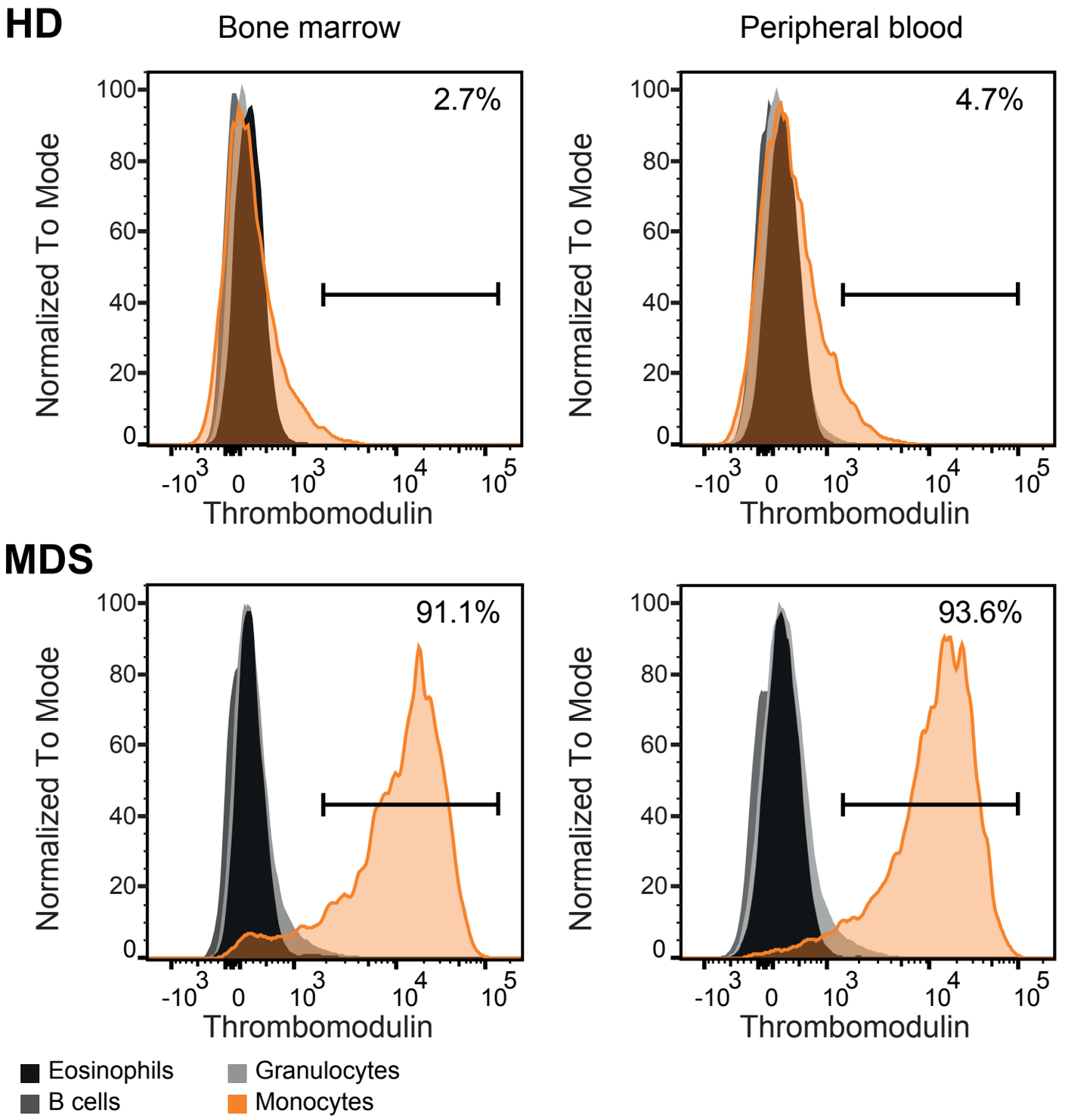


Figure S3. Expression of thrombomodulin on different cell types, including classical monocytes (orange histogram), in bone marrow and peripheral blood of a healthy individual (HD) and a MDS patient. The percentage of TM-positive classical monocytes is shown.

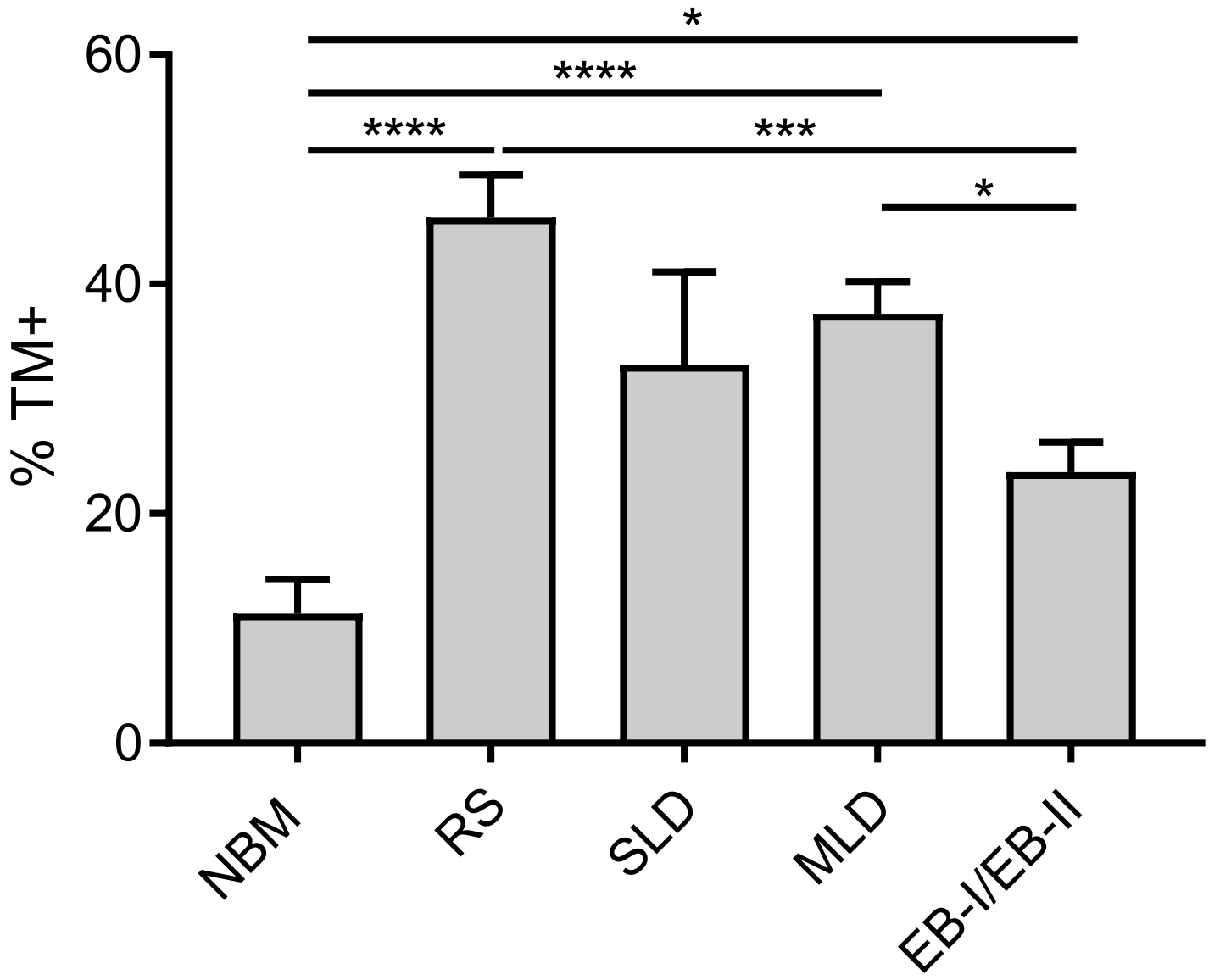


Figure S4. Percentages of TM+ monocytes in WHO 2016 subgroups with and without ring sideroblasts (RS). SLD, single lineage dysplasia; MLD, multilineage dysplasia; EB, excess blasts.

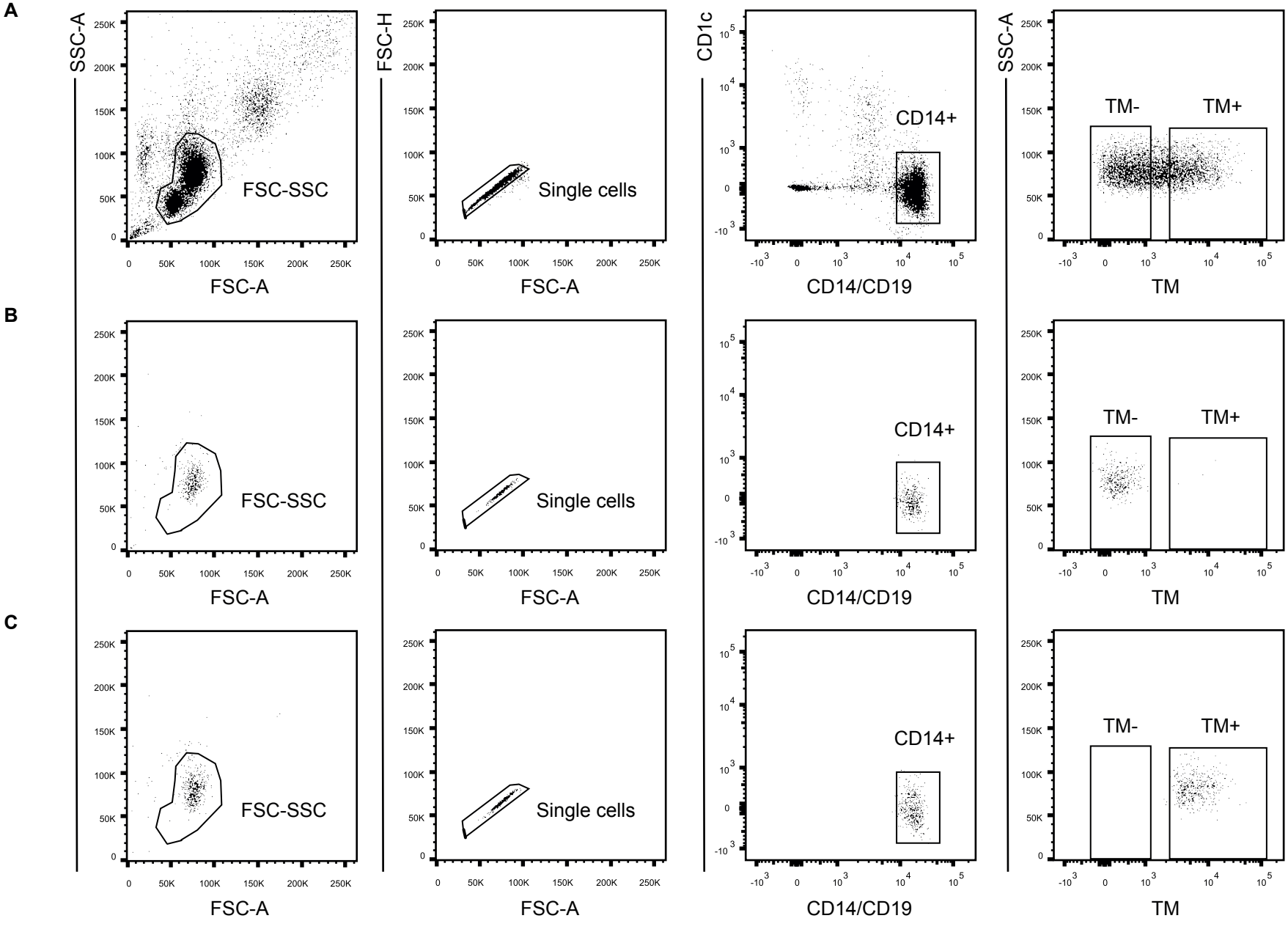


Figure S5. (A) Sort layout for CD14+ monocytes further subdivided in TM- and TM+ monocytes. Purity of sorted TM- (B) and TM+ (C) monocytes was verified afterwards.

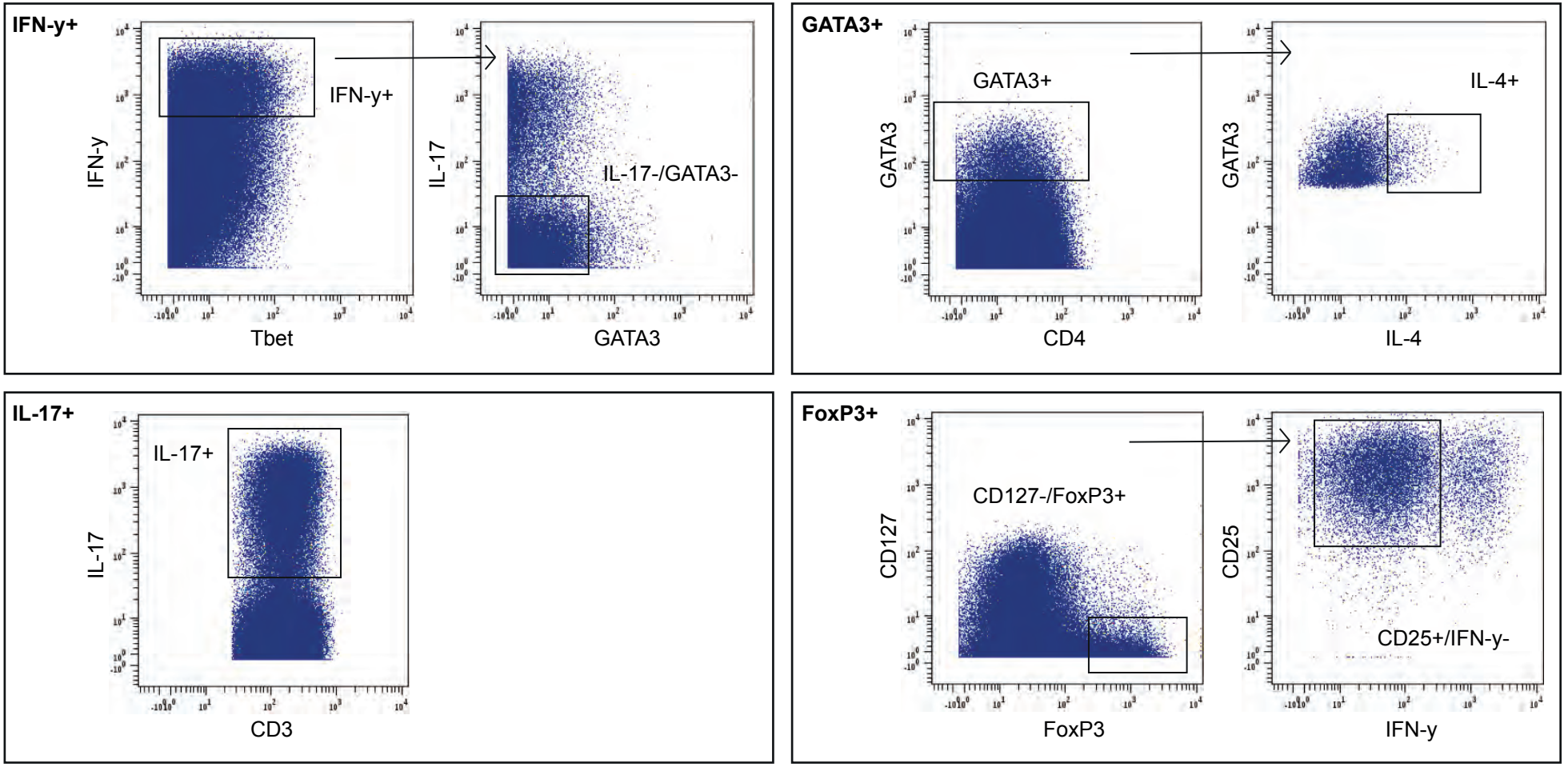


Figure S6. Different T cell subsets were identified for further visualisation in biaxial viSNE plots (shown in figure 3A).

Channel	Target
141	CD3
142	CD19
143	CD56
144	CD11b
145	CD4
147	CD20
148	IL-4
149	CCR4
150	CD62L
151	CD123
152	TNF-a
153	CD45RA
154	CD45
156	IL-6
158	IL-2
159	CD154
160	Tbet
162	CD69
164	IL-17
165	IFN-y
166	CD33
166	CD34
166	CD15
167	GATA3
168	CD8
169	CD25
169	CD25
170	IL-10
171	FoxP3
171	FoxP3
171	FoxP3
172	CD38
174	HLA-DR
175	CD279
176	CD127

Table S1. Antibody panel for mass cytometry staining protocol with cell surface markers, cytokines and transcription factors.